Evaluation of Enzyme-Linked Immunosorbent Assays for Detecting Circulating Antibodies to *Candida albicans*

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Published studies indicate that *Candida albicans* antibody assays utilizing cytoplasmic antigens offer greater utility for identifying cases of systemic candidiasis when compared with assays utilizing cell wall components. We assessed the performance characteristics of a commercially available system that utilizes cytoplasmic antigens to measure *C. albicans* IgG, IgM, and IgA (Candida Detect ELISA reagents). Intra-assay variation was $\leq 5\%$, inter-assay variation was $\leq 10\%$, and good linearity was observed for all the three antibody isotypes. Results for specimens stored under various conditions were comparable to those obtained initially. Inter-laboratory reproducibility was excellent; qualitative

concordance was $\geq 93\%$ for all the three isotypes, with slopes and R^2 values approaching 1.0 in linear regression analyses. Seroprevalence in persons without apparent systemic candidiasis was evaluated using three different serum panels; seroprevalence rates ranged from 24 to 32% for IgG, 2–14% for IgM, and 15–36% for IgA. Seroprevalence rates in a panel of sera containing antibodies to other fungi were similar to rates observed in panels from individuals without systemic candidiasis. These findings demonstrate the acceptable performance of assay systems employing Candida Detect ELISA reagents. J. Clin. Lab. Anal. 22:234–238, 2008. © 2008 Wiley-Liss, Inc.

Key words: candidiasis; *Candida albicans*; IgM; IgG; IgA antibodies; cytoplasmic antigen; ELISA

INTRODUCTION

Systemic candidiasis is an opportunistic disease of the compromised host caused by different species of *Candida*. Conditions associated with candidiasis differ, but can be broadly categorized as (a) nonimmunologic, such as diabetes, trauma, pregnancy, antibiotic therapy, and hyperalimentation (1–5), (b) immunologic, such as malignancies of the immune system (6–9), or (c) immunosuppressive therapy for killing malignant cells (10). Further, oral or esophageal *Candida* infection is found in association with human immunodeficiency virus infection (11).

Although several species of *Candida* cause various clinical forms of candidiasis, *C. albicans* is responsible for the majority of serious cases of systemic candidiasis (10,12,13). However, *C. albicans* may be found as a part of normal flora, especially in the human gastrointestinal tract; thus, the presence of *Candida* does not necessarily indicate systemic disease.

Many reports have shown that serology can be a useful tool for identifying patients with systemic candidiasis. Several types of *Candida* antigens may be used in antibody detection assays, including secreted products, cell wall and membrane contents, and cytoplasmic components. Assays that employ cell wall or membrane antigens detect antibodies in nearly all healthy individuals and persons with commensal *Candida* infections, and are thus of little utility for identifying patients with systemic candidiasis (14–16). In contrast, assays utilizing cytoplasmic antigens show lower seroprevalence rates in healthy individuals, presumably due to low, nonimmunogenic, concentrations in most individuals with nonsystemic infections;

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cytoplasmic antigen-based assays for *C. albicans* antibodies thus appear to offer better utility for the identification of systemic candidiasis patients (16–20). Because it is important that cytoplasmic antigen preparations be free of cell wall and membrane components, methods have been developed that release cytoplasmic antigens while maintaining cell wall integrity (20). More recent publications have described assays that utilize fragments of hyphal cell wall protein 1 (21) or enolase (22) as antigens; however, none of these assays are available commercially.

In this study, we assessed the performance characteristics of a commercially available enzyme-linked immunosorbent assay (ELISA) system for measuring IgG, IgM, and IgA to *C. albicans* cytoplasmic antigen. The parameters evaluated included assay precision and linearity, specimen stability, inter-laboratory reproducibility, prevalence of the different antibody isotypes in sera from apparently healthy individuals, and cross-reactivity in sera containing antibodies to other clinically relevant fungi.

MATERIALS AND METHODS

Patient Specimens

A total of 298 serum specimens, contained within five panels, were used in this study. Table 1 presents the information related to each specimen panel. Panel 2 was tested at Micro Detect, Inc. (MDI, Tustin, CA); all other panels were tested at Focus Diagnostics (Cypress, CA).

TABLE 1. Sera Evaluated Using the Candida Detect IgG, IgM, and IgA ELISA Assays

Panel ID	N	Description
1	76	De-identified sera previously tested using the Candida Detect assays at another laboratory
2	89	De-identified sera submitted to a commercial laboratory for general chemistry testing; assumed to represent patients without systemic candidiasis
3	41	De-identified sera from healthy Focus Diagnostics employees; assumed to represent individuals without systemic candidiasis
4	58	De-identified sera submitted to Focus Diagnostics for rabies virus antibody measurement; assumed to represent healthy subjects, without systemic candidiasis, who received the rabies virus vaccine due to potential occupational exposure
5	34	De-identified sera positive for antibodies to <i>Aspergillus</i> , <i>Blastomyces</i> , <i>Coccidioides</i> , or <i>Histoplasma</i> by immunodiffusion testing (cross-reactivity panel)

ELISA Method

Serum IgM, IgA, and IgG antibodies to *C. albicans* cytoplasmic antigens were measured using Candida Detect IgM, IgG, and IgA ELISA reagents obtained from Micro Detect, Inc. Specimens were routinely tested at a final dilution of 1:200. For IgM assessment, sera were pre-diluted 1:10 in diluent containing goat anti-human IgG, incubated at room temperature (RT) for 20 min, and then further diluted 1:20 in routine specimen diluent. Diluted sera were added to microtiter wells, followed by RT incubation for 20 min. After washing, wells received conjugate (horseradish peroxidase-tagged goat anti-human IgG, IgA, or IgM), and were incubated at RT for 20 min. After washing, wells received substrate (tetramethylbenzidine), and after 15 min at RT, color development was stopped by the addition of dilute sulfuric acid. Absorbance at 450 nm was measured, and the results were expressed as an index, calculated by dividing the specimen absorbance value by the mean absorbance value obtained for a calibrator serum included in each assay run. Index values <1.00 were considered negative, and ≥ 1.00 positive.

Linearity

Linearity of Candida Detect IgG, IgM, and IgA assays was determined at MDI by measuring the absorbance values of two-fold serial dilutions of sera positive for *C. albicans* IgG, IgA, or IgM. Dilutions spanned the range 1:128–1:2048.

Precision

Intra-assay precision was determined at MDI by two operators who tested 16 replicates of two sera. Intra-assay precision was determined at Focus Diagnostics by one operator who tested eight replicates of three sera. Inter-assay precision was determined at Focus Diagnostics by one operator who tested nine sera on 5 separate days.

Sample Stability Studies

Sample stability was evaluated at Focus Diagnostics by testing aliquots of nine samples (the same samples used for inter-assay precision studies) stored under four different conditions: (a) 7 days at RT, (b) 14 days at 4°C, (c) 30 days at –70°C, and (d) subjected to four freeze–thaw cycles over 14 days (freezer temperature –70°C).

RESULTS

Assay Linearity

Absorbance values for dilutions of sera positive for *C. albicans* IgG, IgM, or IgA are shown in Figure 1.

With increasing dilution, absorbance values decreased in a linear fashion. Based on these findings, a serum dilution of 1:200 was selected for routine use in all the three assays.

Precision

Table 2 shows intra-assay variation for Candida Detect IgG, IgM, and IgA assays as determined at both MDI and Focus Diagnostics. Coefficient of variation (CV) values ranged between 1 and 5%. Table 3 shows inter-assay variation data for nine specimens as determined at Focus Diagnostics; CV values ranged between 3 and 10%.

Specimen Stability

Results of studies assessing stability for nine specimens under various storage conditions demonstrated excellent stability. All results for stored samples were qualitatively concordant with original results; further, index values for all stored specimens were within 26% of the original index value.

Fig. 1. Linearity of Candida Detect IgG, IgM, and IgA assays.

TABLE 2. Intra-Assay Precision^a

Site	Replicates	Sample 1			Sample 2			Sample 3		
		IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
MDI (operator 1)	16	0.86 (4%)	0.76 (3%)	1.16 (2%)	0.29 (4%)	0.22 (4%)	0.59 (3%)	nd	nd	nd
MDI (operator 2)	16	0.84 (3%)	0.77 (2%)	1.18 (2%)	0.30 (3%)	0.22 (4%)	0.59 (3%)	nd	nd	nd
Focus Diagnostics	8	1.64 (1%)	2.26 (3%)	2.03 (3%)	1.27 (1%)	1.41 (4%)	1.50 (3%)	0.33 (2%)	0.69 (5%)	0.17 (4%)

Note: Samples 1 and 2 used at MDI were different from Samples 1 and 2 used at Focus Diagnostics. nd = not determined, ELISA, enzyme-linked immunosorbent assay.

^aMDI results represent mean absorbance (CV); focus results represent mean index (CV).

Inter-laboratory Reproducibility

Panel 1 was supplied by Quest Diagnostics (San Juan Capistrano, CA), where 39 of 76 specimens were IgG+, 21 of 76 were IgM+, and 28 of 76 were IgA+ when tested using the Candida Detect assays. Results obtained for this panel when tested at Focus Diagnostics were quite comparable to the results obtained at Quest Diagnostics (Table 4). Qualitative concordance was ≥93% for all the three isotypes. Of the 14 qualitatively discordant result sets (four IgG, five IgM, five IgA), ten results sets (three IgG, four IgM, four IgA) exhibited a Focus index result within 25% of the Quest result, indicating good agreement even though they differed

TABLE 3. Interassay precision*

Specimen ID	Candida Detect IgG (%)	Candida Detect IgM (%)	Candida Detect IgA (%)
1	1.56 (7)	1.94 (4)	1.72 (6)
2	1.53 (5)	2.66 (3)	1.84 (5)
3	1.25 (8)	1.36 (5)	1.43 (7)
4	1.33 (6)	1.28 (3)	2.50 (6)
5	1.20 (4)	1.43 (4)	1.62 (9)
6	1.65 (5)	2.11 (7)	2.01 (6)
7	0.34 (8)	0.66 (4)	0.17 (4)
8	0.27 (7)	0.14 (7)	0.15 (7)
9	0.31 (10)	0.52 (3)	0.29 (10)

*Results represent mean index (CV) for five determinations, each performed on a different day. CV, coefficient of variation.

TABLE 4. Inter-Laboratory Reproducibility^a

Parameter	Candida Detect IgG	Candida Detect IgM	Candida Detect IgA
Qualitative concordance	95%	93%	93%
Linear regression slope	0.947	1.039	1.130
Linear regression R ² value	0.982	0.970	0.966

^aSamples were those contained in panel 1 (N = 76).

TABLE 5. Prevalence of *C. albicans* Antibodies in Different Panels of Sera From Individuals Assumed to be Free of Systemic Candidiasis

Panel number	N	Proportion IgG+ (%)	Proportion IgM+ (%)	Proportion IgA+ (%)
2 (chemistry tests)	89	25	2	15
3 (employees)	41	32	5	32 ^a
4 (rabies antibody tests)	58	24	14 ^a	36 ^a

^aSignificantly different from the comparable value for group 2 (significance defined as $P < 0.05$, chi-squared test for differences among proportions).

qualitatively. In linear regression analyses, slopes were near 1.00 (range 0.95–1.13) and R^2 values were > 0.96 for all the three isotypes.

Prevalence of *C. Albicans* Antibodies in Panels of Sera Individuals Without Systemic Candidiasis

Initial assessment of seroprevalence rates in individuals assumed to be free of systemic candidiasis was performed at MDI using panel 2 (de-identified sera submitted to a commercial laboratory for routine chemistry tests). As shown in Table 5, the observed seroprevalence was highest for IgG, followed by IgA, then IgM. Panel 3 (healthy employee sera) was tested at Focus Diagnostics as part of assay verification studies; although the IgG and IgM seroprevalence rates for panel 3 were not significantly different from those observed for panel 2, the IgA seroprevalence rate for panel 3 was significantly higher than for panel 2 (32 vs. 15%, respectively, see Table 5). Based on this difference, an additional serum panel (panel 4, sera submitted for rabies antibodies, see Table 1 for description) was evaluated at Focus Diagnostics. Both the IgM and IgA seroprevalence rates for panel 4 were significantly higher than those observed for panel 2; however, none of the panel 4 seroprevalence rates were significantly different from those observed for panel 3 (Table 5). Based on the similarity of seroprevalence rates in the two panels of sera from healthy individuals tested at Focus Diagnostics (panels 3 and 4), data for these panels were combined to yield seroprevalence rates of 27% for IgG, 10% for IgM, and 34% for IgA.

Cross-Reactivity

To assess potential cross-reactivity associated with other fungal infections, a set of 34 sera (panel 5) positive for antibodies to *Aspergillus*, *Blastomyces*, *Coccidioides*, or *Histoplasma* were tested in Candida Detect IgG, IgM, and IgA assays at Focus Diagnostics. The seroprevalence rates for this panel were then compared with those observed for the combined healthy individual panel

TABLE 6. Seroprevalence of *C. albicans* Antibodies in the Cross-Reactivity Panel

Panel ID	N	Proportion IgG+ (%)	Proportion IgM+ (%)	Proportion IgA+ (%)
Combined healthy individual panel	99	27	10	34
Panel 5 (cross-reactivity)	34	41	21	35

(panel 3 plus panel 4). As shown in Table 6, for all three *C. albicans* antibody isotypes, the seroprevalence rate observed for the cross-reactivity panel (panel 5) did not significantly differ from the rate observed for the combined healthy individual panel.

DISCUSSION

The Candida Detect IgG, IgM, and IgA ELISAs demonstrated very good performance, as indicated by acceptable linearity, low intra-assay and inter-assay CV values, and robust specimen stability. Inter-laboratory reproducibility, as demonstrated by testing of a large panel of sera at two different locations, was also excellent, confirming the robust nature of these ELISAs. Seroprevalence rates in a panel of sera positive for antibodies to other fungal pathogens were similar to those observed in healthy individuals, indicating that cross-reactivity due to other fungal infections is not a serious issue with the Candida Detect antibody assays.

The detection of antibodies to *C. albicans* cytoplasmic antigens in a portion of healthy individuals complicates the utilization of these assays for identifying patients with systemic candidiasis. It is thus important to determine the expected seroprevalence rates for *C. albicans* IgG, IgM, and IgA in healthy individuals. Our findings demonstrate the challenges in making these determinations. Among the three different panels representing individuals without systemic candidiasis, the IgG seroprevalence rate was consistent at 24–32%; however, differences were observed in the seroprevalence rates for IgM (range 2–14%) and IgA (range 15–36%). The factors contributing to these differences remain unclear, and may include age, gender, geographic area of residence, and/or lifestyle-related fungal exposure. Large well-controlled studies are thus needed to firmly establish the seroprevalence rates of *C. albicans* antibody isotypes in the general population.

Likewise, studies assessing the clinical utility of *Candida* antibody assays are sorely needed; although several small studies have addressed this topic over the last 25 years, well-controlled studies using sera from large numbers of well-characterized patients with systemic candidiasis are lacking. Although we sought

such samples for inclusion in this study, our search was unsuccessful. Of particular interest would be a direct comparison of assays utilizing cytoplasmic proteins, hyphal cell wall protein 1 fragments, and enolase as target antigens. Until such studies are performed, the sensitivities, specificities, and predictive values of *Candida* antibody assays remain unclear.

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